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THE RELATIONSHIP BETWEEN pH AND THE ACTIVITY OF CHOLINESTERASE FROM FLIES

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In order to make useful comparisons of similar enzyme systems from different organisms, or of the response of a given system to different chemical agents, some understanding of the possible effect of changes in the conditions of assay is essential. This requirement becomes especially conspicuous in attempts to correlate results from laboratories whose techniques are not identical, as illustrated, for example, by certain discrepancies that will be discussed below. In commencing a series of studies intended to bring to light the distinctive properties of insect cholinesterases (ChE's), we have therefore found it expedient to examine in detail the effects of altering our experimental conditions, and have already reported the results of tests in which the activity of fly head ChE was measured in relation to the composition of the suspending medium (Chadwick, Lovell and Egner, 1953).

Another factor with significant influence *in vitro* on the rate of hydrolysis of acetylcholine (ACh) by ChE is the hydrogen ion concentration. For most ChE's that have been studied in this connection, enzymic activity was maximal somewhat on the alkaline side of neutrality, fell rather sharply at still higher pH values, and declined more gradually as hydrogen ion concentration was increased. The pertinent references are discussed by Augustinsson (1948); see also Table IV below.

Three studies of the problem with insect material have been reported. Tahmisian (1943) found a relationship of typical form and a pH optimum of 8.5 with the enzyme from developing grasshopper eggs. Stegwee (1951), working with central nervous tissue of the beetle, *Hydrophilus,* and the roach, *Periplaneta,* recorded rather sharp optima at pH 7.4. Data of Babers and Pratt (1950) with fly head suspensions are in contrast with these and all other reports in that they indicate a peak in activity at about pH 5.75. In their preparations, ChE activity was maintained near half peak level between pH 6.25 and 9.00, and decreased abruptly at higher pH values, as at values below 5.50. They comment (p. 61) that "this activity over such a wide pH range not only was unexpected but is also unexplained"; however, the most unusual feature of their results is the position of the optimum.

At the time their work was published, our investigations of the same problem were already in progress, and it was apparent immediately that our observations did not agree fully with theirs. We therefore extended the scope of the experiments, first in order to establish more firmly the nature of the relationship between

pH and the activity of fly head ChE, and secondly with the hope of reconciling the differences between our data and those of Babers and Pratt. In addition, since the reduction in activity at low and high pH was found to involve some irreversible inactivation of the enzyme, we made measurements of this aspect of the process.

Experimental

Culture of flies (*Musca domestica* L.) preparation of head suspensions, and our application of Glick's (1937) titrimetric method of measuring ChE activity have been described in an earlier report (Chadwick, Lovell and Egner, 1953). In the present experiments, data were obtained at 25.0 degrees C. on 20-ml. aliquots, containing the equivalent of 20 heads each, with three suspension media. Of these, the first was buffer: NaCl, 26.30 gm.; KH_2PO_4, 3.85 gm.; NaOH, 1.00 gm.; H_2O, to one liter. This solution was designed to promote maximal enzymic activity, which had been found to require the presence of a salt at about 0.5 N concentration; and was buffered lightly, so as to minimize fluctuation of pH during assay and yet retain sufficient sensitivity for accurate determination of the rate of production of acid. Since our results with this medium differed considerably from those reported by Babers and Pratt (1950), whose suspensions contained glycerol, a second series of observations was made with head tissue ground and assayed in 30 per cent glycerol. In a third set of experiments, the brei was suspended in de-ionized water. With all the suspensions, pH was adjusted to the desired level by addition of NaOH or HCl.

The total acid production during test periods of approximately 15 minutes was corrected by subtraction of the acid produced under conditions that were identical except that the enzyme had been inactivated by exposing the stock brei overnight or for a longer time to 1×10^{-5} *M* diisopropyl fluorophosphate (DFP). The net, or enzymic, activity was then converted into micromoles of ACh.Br hydrolyzed per ml. (*i.e.*, per head) per hour.

The rate of permanent inactivation of fly head ChE at low and high pH was determined on aliquots that were incubated at the desired pH value for definite periods of time, and then readjusted rapidly to pH 8.0 before addition of ACh.Br for assay.

In all these experiments, the concentration of substrate at the beginning of measurement was 0.015 *M*. Other concentrations were used in a few experiments for special purposes, as cited in the discussion.

Results

Average rates of enzymic hydrolysis of 0.015 *M* ACh.Br at various pH values in the three media tested are given in Table I. The data have been plotted in Figure 1 as percentages of the average value determined for these tissue samples in buffer at pH 8.0.

Also shown in Table I are the corresponding corrections for non-enzymic hydrolysis. These were evidently not identical in the several media. As pointed out in our previous paper, such variation results in part from the fact that pH

is not truly constant in our method of measurement. Each addition of NaOH during titration pushes pH to the alkaline side of the chosen value, and, for a given amount of alkali, such excursions are greater the more weakly buffered the solution. Error from this cause is not overly significant at pH 8.0 and below, but increases rapidly in more alkaline solutions, where the rate of non-enzymic hydrolysis of ACh is rising steeply with increase in pH. Since ChE activity, as measured in well buffered solutions, increases but slightly above pH 8.0, the error resulting from fluctuation of pH in the experimental samples is largely in the nonenzymic fraction and should theoretically be compensated by the nearly equal error in the controls. In practice, however, we found it difficult to obtain satisfactorily consistent results at pH 9.5 and 10.0.

In order to supply some indication of the range of variation encountered, we have computed standard errors for the means in each series except for those

TABLE I

Enzymic activity of fly head ChE as a function of pH

pH	4.0	5.0	5.5	6.0	7.0	7.5	8.0	8.5	9.0	9.5	10.0
In buffer											
Net rate*	nil	0.34	1.97**	2.05	4.05	4.39	4.88	5.08	5.27	4.64	1.17
±s.e.	—	0.03	—	0.13	0.18	0.11	0.10	0.24	0.22	0.36	0.83
n***	4	10	5	9	10	11	31	10	13	10	10
Correction	—	—	−0.03	−0.03	−0.07	−0.09	−0.24	−0.56	−1.52	−4.78	−13.60
In de-ionized water											
Net rate*	nil	0.54	1.42	1.66	2.24	2.54	2.15	1.66	2.88	1.46	0.88
±s.e.	—	0.07	0.18	0.24	0.21	0.24	0.12	0.16	0.51	—	—
n***	6	10	10	10	10	10	17	10	10	5	5
Correction	—	—	−0.01	−0.02	−0.04	−0.28	−0.56	−1.21	−3.10	−7.63	−22.17
In glycerol, 30 per cent											
Net rate*	nil	0.44	0.88	1.27	1.42	0.98	1.37	0.88	1.51	0.63	0.15
n***	5	5	5	6	5	5	13	5	5	5	5
Correction	—	—	—	−0.01	−0.02	−0.25	−0.54	−1.14	−2.63	−4.24	−7.25

* Average net rates, standard errors, and corrections in micromoles ACh.Br hydrolyzed per ml. (= per head) per hour.

** pH, 5.75.

*** n, number of tests.

All runs at 25.0 degrees C.; ACh.Br, 0.015 *M*.

where only 5 determinations were made at each pH level. We report these calculations with some hesitation, first because of the relatively small "n," and secondly because the measurements at different pH values in a single series were not wholly independent. For example, aliquots of a stock brei which showed more than average activity at one pH value tended to give higher than average measurements at all pH levels. Thus, the sampling was not truly random, and on this account the standard errors listed in the table should not be relied on for estimates by the t-test of the significance of differences between means.

The second table shows the average activity remaining in samples that had been incubated at the indicated pH values for 30 minutes and then readjusted to pH 8.0 for assay. In Table III are recorded the results of exposing samples to pH 4.0 for different periods of time, up to two hours. Some few additional data pertinent to these experiments are cited in the discussion.

Table VI shows ChE activity as a function of pS at two pH levels, *viz.*, 6.0 and 8.0.

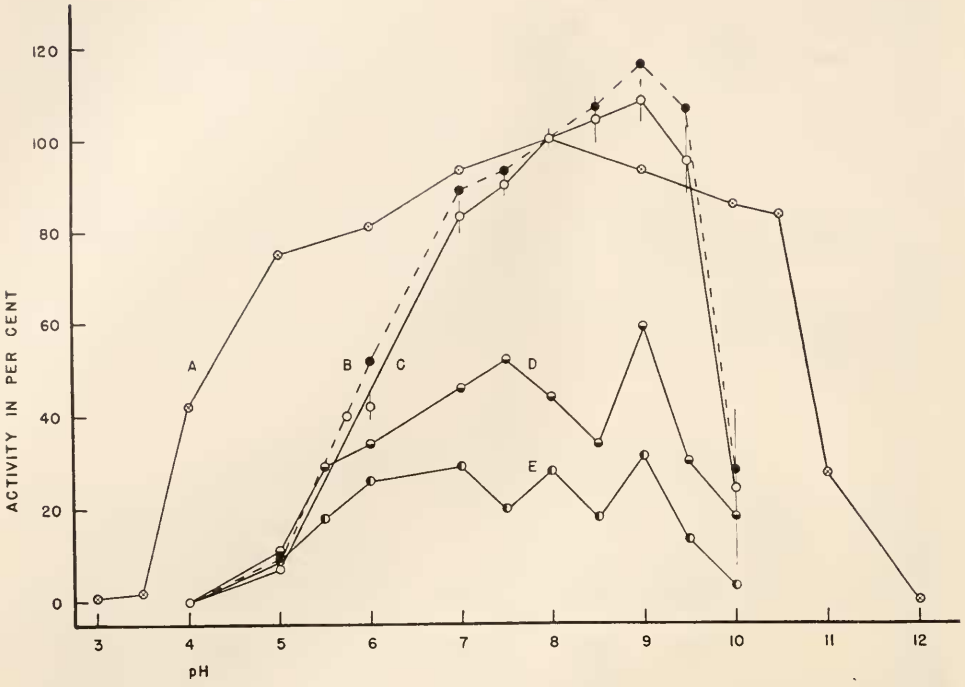

FIGURE 1. Variation in ChE activity of fly heads as a function of pH. Curve A. Residual activity after 30-minute exposures at indicated pH values; all measurements at pH 8.0 in buffer. Curve B. Specific activity at indicated pH values, computed by correcting Curve C for degree of inactivation shown in Curve A. For fuller explanation, see text. Curve C. Activity measured during 15-minute exposures at indicated pH values in buffer. The open circles give the mean values, and the vertical bars indicate the limits for ± 3 s.e. Curve D. Activity measured during 15-minute exposures at indicated pH values in water. Curve E. Activity measured during 15-minute exposures at indicated pH values in 30 per cent glycerol. All data shown have been corrected for non-enzymic hydrolysis.

TABLE II

ChE activity of fly head suspensions after 30 minutes exposure at various pH values

pH	Average enzymic activity (per cent)	Number of observations	pH	Average enzymic activity (per cent)	Number of observations
3.0	1.0	1	8.0	100.0	10
3.5	1.9	5	9.0	93.2	5
4.0	41.9	4	10.0	85.6	5
5.0	75.0	4	10.5	83.2	5
6.0	81.1	5	11.0	27.2	5
7.0	93.4	5	12.0	nil	2

All runs at 25.0 degrees C.; ACh.Br, 0.015 *M*; samples readjusted to pH 8.0 for measurement.

TABLE III

Rate of inactivation of fly head ChE at pH 4.0 as a function of duration of exposure

Time exposed minutes.....	0	15	30	60	90	120
Activity in per cent	100	65.9	41.9	30.1	24.9	20.5
Number of observations	20	5	4	4	4	4

All runs at 25.0 degrees C.; ACh.Br, 0.015 *M*; samples readjusted to pH 8.0 for measurement

DISCUSSION

1. *ChE activity as a function of pH*

Examination of the measurements in buffer convinces us that the ChE of our fly heads differs little, in respect to the effect of pH on activity, from most other ChE's hitherto studied (*cf.* Table IV). The optimum is clearly on the alkaline side, being at least as high as 8.0 and probably as high as 9.0.

In aqueous suspensions or in 30 per cent glycerol, activity was generally low in comparison with observations at corresponding pH values in buffer, with the rates in glycerol somewhat less than those in water. These data provide a further demonstration of the activating effect of 0.5 N salt and the depressant effect of glycerol, to which we called attention earlier (1953). In water or glycerol there appeared to be little significant change in ChE activity over the pH range from

TABLE IV

pH optima of ChE's from various sources

Source of enzyme	pH optimum	Authority
Eggs, developing, *Melanoplus*	8.5	Tahmisian, 1943
Erythrocytes, human	7.6 or above	Plattner *et al.*, 1928
Erythrocytes, human	7.5 to 8.0	Alles and Hawes, 1940
Serum, human	8.2 or above	Plattner *et al.*, 1928
Serum, human	8.0 or above	Easson and Stedman, 1936
Serum, human	8.4 to 8.5	Glick, 1937
Serum, human	8.0 to 8.5	Werle and Uebelmann, 1938
Serum, horse	ca. 8.5	Glick, 1938
Serum, horse	7.2 or above	Kahane and Levy, 1936
Serum, horse	8.0 to 8.5	Werle and Uebelmann, 1938
Heart extract, frog	7.5 or above	Loewi and Navratil, 1926
Electric organ, *Electrophorus*	ca. 8.5	Wilson and Bergmann, 1950
Gastric mucosa, pig	ca. 8.5	Glick, 1938
Brain, rat (also rabbit, guinea pig, cat, dog)	ca. 8.4	Bernheim and Bernheim, 1936
Brain, cat	ca. 8.5	Glick, 1938
c.n.s., *Periplaneta*, *Hydrophilus*	7.4	Stegwee, 1951
Heads, *Musca*	5.75	Babers and Pratt, 1950
Heads, *Musca*	8.0 or above	This paper

6.0 to 9.0, in agreement with the findings of Babers and Pratt (1950); however, variation in our measurements was considerable and the curves are quite irregular.

Neither in these media nor in buffer could we find any evidence for an activity peak in the neighborhood of pH 5.75, as reported by Babers and Pratt. This led us to attempt one final comparison, in which activity at pH 5.75 and 7.0 was measured under conditions as nearly like theirs as we could make them. For this purpose, tissue was prepared in 30 per cent glycerol and diluted 1:6 for assay, which was carried out on 9.0-ml. aliquots that contained 150 mg. of tissue and 0.045 *M* ACh.Br. Babers and Pratt had used 3.0-ml. samples containing 50 mg.

Table V

Comparison of ChE activity of fly head suspensions in 5 per cent glycerol at pH 7.0 and 5.75

	pH 7.0	pH 5.75
	ml. 0.02 N NaOH per 3 ml. per 20 minutes	
	1.00	1.05
	1.05	1.03
	0.98	1.09
	1.03	1.02
	1.01	1.01
Average	1.01	1.04
*Correction	−0.04	−0.01
Net	0.97	1.03

* Correction values from Babers and Pratt (1950). All runs at 25.0 degrees C.; ACh.Br, 0.045 *M*; tissue, 150 mg.; total volume, 9.0 ml. Data computed to 3.0 ml. volume for sake of comparison with results of Babers and Pratt.

of tissue, but this volume was too small for our electrodes. As in their tests, acid produced was titrated with 0.02 N NaOH over a 20-minute test period. Five replications were made. The results, corrected for non-enzymic hydrolysis with values taken from Babers and Pratt (1950), are shown in Table V.

These data suggest the following comments. First, activity was nearly equal at both pH values; *i.e.*, evidence for a pronounced peak at pH 5.75 was not forthcoming. Secondly, as was to have been expected, activity per unit weight of tissue, or per head, was intermediate at pH 7.0 between the values previously found with suspensions in water and in 30 per cent glycerol, respectively. Finally we may

Table VI

Activity of fly head ChE as a function of substrate concentration at two pH levels

Molar concentration of ACh.Br	0.001	0.003	0.01	0.03	0.10
	Average enzymic activity in micromoles per head per hour				
pH 8.0	4.83	5.11	5.29	4.51	2.83
pH 6.0	1.22	1.77	2.37	2.44	1.71

Each datum is the mean of 5 determinations. All runs on aliquots of the same stock brei in buffer at 25.0 degrees C.

note that the activity of our preparation, at both pH levels, was more than twice the peak value reported by Babers and Pratt (1950). Unless some undetected difference in our methods of preparing the tissue can be held responsible, this observation indicates a possible strain difference between their flies and ours; and should strain differences of this magnitude exist, they could conceivably extend to a shift in the pH optimum from above 8.0 to 5.75. This, however, seems very unlikely in view of the bulk of evidence (Table IV) in favor of an alkaline pH optimum for ChE's in general. The remaining alternative is to ascribe the observation of Babers and Pratt to fortuitous variation in the activity of different breis; *i.e.,* to a somewhat unlikely coincidence of sampling errors, that led them repeatedly to exceptionally high values at pH 5.75. This solution does not appeal to us, since it is obviously indemonstrable and because the same sort of inference could, with equal justification, be applied to our own data; but all our efforts to find a more satisfactory explanation have failed.

Theoretical reasons for anticipating an increase in optimal concentration of substrate as conditions of measurement depart from the pH optimum have been put forward by Wilson and Bergmann (1950). The data in Table VI bear on this question, and do in fact indicate a slight shift of $pS_{opt.}$ in the predicted direction at pH 6.0 as compared with pH 8.0. Although it is of interest that this shift should appear in our results, the presence of the effect will hardly demand correction of the pH-activity data in Table I and Figure 1, for the following reasons. The magnitude of the shift is small, the optima are relatively flat, and the standard concentration of 0.015 *M* ACh.Br used routinely in our experiments is already somewhat above the optimum for pH 8.0.

2. *Inactivation of fly head ChE*

The reduction in activity of fly head ChE at hydrogen ion concentrations that depart appreciably from pH 8.0 is not wholly reversible. This fact raises a question as to what portion of the activity change observed at different pH levels is due to an effect of pH on reaction rate, and what portion to permanent destruction of a fraction of the enzyme. Obviously, data such as those in Table I must reflect a summation of both these processes.

We have attempted in a preliminary manner to separate the two effects by measuring the irreversible inactivation of ChE that results when the suspensions are exposed in buffer to different pH values for a constant period of time. The interval chosen was 30 minutes, this being somewhat longer than the average total exposure during our routine 15-minute determinations. As indicated in Table II, the percentage inactivation observed under these conditions remained within moderate limits until one passed below pH 5.0 or above pH 10.5. Other observations not given in the table showed that, within this pH range, there was little if any additional loss of activity during exposures of as much as two hours; and further that subsequent incubation of the samples at pH 8.0 for as long as 18 hours caused no reversal of the loss that had already occurred.

That inactivation did not take place instantaneously was demonstrated by a series of tests at pH 4.0, where suspensions were held for periods varying from 15 minutes to two hours, before return to pH 8.0 for assay (Table III). Here

the process of inactivation was rapid for the first 30 minutes, and followed a slower course thereafter. Both segments of the relationship have the characteristics of a first order reaction, as indicated in Figure 2.

Below pH 4.0 and above pH 11.0, inactivation was rapid and extensive. As a matter of fact, the enzyme, together with large amounts of eye pigment, was precipitated from aqueous suspensions of head tissue at about pH 5.0 to 5.1. Some 75 per cent of the original activity could be recovered if this precipitate

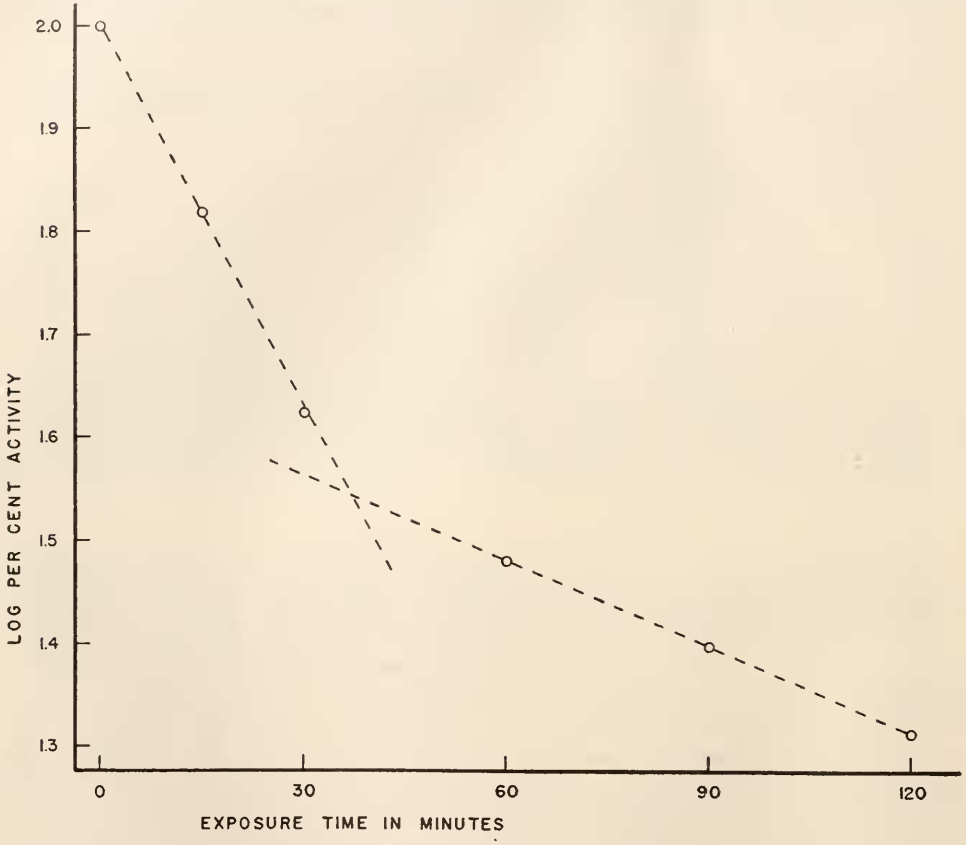

FIGURE 2. Rate of inactivation of fly head ChE in buffer as a function of duration of exposure at pH 4.0. All measurements made at pH 8.0. For further details, see text and Table III.

was quickly re-suspended in buffer at pH 8.0; but it was evident that the enzyme had been altered, since it was no longer as soluble as before precipitation. It was now easily re-separated by light centrifugation. According to Augustinsson (1948), the isoelectric point of several other ChE's has been reported as about pH 4.5. He also notes that precipitation at this level results in more or less permanent solubility changes.

By combining the data of Table II, which show the fraction of enzyme still active after half-hour exposures at the various pH values, with those of Table I,

which give the rates at which similar aliquots were able to hydrolyze substrate while at the same pH levels, it is possible to construct a corrected curve that compensates for changes in the relative amount of active enzyme. Such a curve is shown as B in Figure 1. The divergence from Curve C, which embodies the uncompensated activity data, is slight. This is because 30-minute exposures at pH values between 5.0 and 10.5 inactivated only small fractions of the enzyme, while the effect of pH on reaction rate was already considerable, well within these limits. The compensated curve (B) emphasizes the activity peak at pH 9.0, since the proportion of active enzyme has already begun to fall in this region, whereas the measured activity per unit of tissue has increased slightly above the value determined at pH 8.0. The results also show incidentally that the drop in activity at still higher pH levels is not due wholly to denaturation of the enzyme, since the measured activity decreased more rapidly than the enzyme was destroyed.

These data suggest a pH of about 8.0 as a suitable compromise for experiments where an approach to maximal activity of fly head ChE is desired. Although the true optimum probably lies as much as a full pH unit to the right of pH 8.0, the increase in enzymic activity over this range is slight, whereas the correction for non-enzymic hydrolysis is rapidly becoming larger. At pH 8.0, this correction is less than 5 per cent of the average total activity measured under our conditions; *i.e.,* with tissue concentration at one head per ml.; temperature, 25.0 degrees C.; substrate, 0.015*M*; and salt present in the buffered suspension at about 0.5 N concentration. The correction could be still further reduced by shifting to even lower pH levels, but only with increasing sacrifice of enzymic activity.

Summary

1. Variation in activity of fly head cholinesterase (ChE) was measured titrimetrically at 25.0 degrees C. with ACh.Br 0.015 *M* as substrate, as a function of the pH of the assay medium over the range from pH 4.0 to 10.0. Ground tissue obtained from *Musca domestica* L. was suspended at a concentration of one head per ml. in three media: (1) buffer of composition NaCl, 26.30 gm.; KH_2PO_4, 3.85 gm.; NaOH, 1.00 gm.; H_2O, to one liter; (2) 30 per cent glycerol; (3) de-ionized water.

2. Enzymic activity was greater in buffer than in the other media. The pH optimum was definitely on the alkaline side, being at least as high as pH 8.0 and probably as high as 9.0. In glycerol or water suspensions, enzymic activity changed little between pH 6.0 and 9.0.

3. Some permanent inactivation of the enzyme was observed in half-hour exposures at high and low pH values. This effect was measured over the pH range from 3.0 to 12.0. Between pH 5.0 and 10.5, the degree of inactivation was moderate and essentially complete within 30 minutes. The time course of the process was followed at pH 4.0 for intervals from 15 minutes to two hours, and appeared to involve a rapid phase during the initial 30 minutes and a slower phase thereafter. Both phases had the characteristics of a first order reaction. Inactivation of ChE resulting from exposure to low or high pH was not reversed during subsequent incubation of the sample at pH 8.0 for as long as 18 hours.

4. Correction of the pH-activity curve to allow for changes in the relative amounts of enzyme that result from permanent inactivation requires only minor

alterations, since the effect of pH on reaction rate makes itself felt within pH limits where the degree of permanent inactivation is slight.

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